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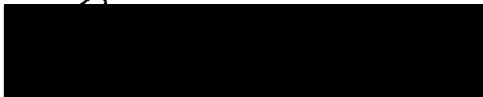
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AN ABSTRACT OF THE THESIS OF Michael E. Darling for the
Master of Science in Biology presented July 29, 1971.

Title: Lactic Dehydrogenase Isozyme Isolation by Disc
Electrophoresis in Eight Species of Fringillid Birds.

APPROVED BY MEMBERS OF THE THESIS COMMITTEE:


Richard B. Forbes, Chairman


W. Herman Taylor


Mary L. Taylor


John H. Wirtz

Disc electrophoresis of tissue homogenates from eight species of fringillid birds showed five forms of lactic dehydrogenase. The relative amounts of isozymes were characteristic for each species. This paper classifies some members of the families Fringillidae and Carduelidae in terms of lactic dehydrogenase isozymes and compares the results to other methods of classification.

LACTIC DEHYDROGENASE ISOZYME ISOLATION BY DISC ELECTROPHORESIS IN
EIGHT SPECIES OF FRINGILLID BIRDS

BY

Michael Edwin Darling

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE

IN

BIOLOGY

Portland State University

1971

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July 29, 1970

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INTRODUCTION

Birds and mammals have five multiple molecular forms of lactic dehydrogenase (LDH). The multiple molecular forms found in a given species are called isozymes. Each isozyme can be separated into four subunits of the same molecular weight. The four subunits can be separated into two classes of polypeptide chains, H and M. The isozyme designated M_4 consists of four M chains. The isozyme designated H_1M_3 consists of three M chains and one H chain. The isozyme designated H_2M_2 consists of two M chains and two H chains. The isozyme designated H_3M_1 consists of one M chain and three H chains. The isozyme designated H_4 consists of four H chains (Lehninger, 1970). All isozymes have the same molecular weight and carry out the same overall reactions, but differ in chemical structure, immunological properties, shape, and charge. The H_4 isozyme is found to predominate in the heart muscle and is strongly inhibited by a high pyruvate concentration. Ratite birds and poor fliers have the M type in their breast muscles, whereas stronger fliers have a much larger percentage of the H type (Fine and Costello, 1962). Pyruvate in fatty acid metabolism is converted into lactate. H_4 isozyme may be necessary in the control of this pathway (Fine and Costello, 1962). The M_4 isozyme predominates in the white skeletal muscle and is weakly inhibited by high pyruvate concentrations (Bailey and Wilson, 1968).

All the cells of an animal have the capacity to produce

combinations of both types of LDH, but there are unknown mechanisms involved in its differentiation and development. Each organ possesses a different quantitative amount of each type (Fine and Costello, 1962). According to the subunit hypothesis, LDH isozymes are composed of two subunits, A and B in varying combinations of four. According to Vesell and Brody (1964), each subunit is under the control of two separate genes, a and b. The differences in the relative activity of these genes from one tissue to another and the random recombination of the subunits produced by the genes provides the explanation of the variation in isozyme pattern in specialized cells of the same organism.

The homologies of LDH among classes of chordates are complex; however, a taxonomic study of LDH isozymes homologies may lead to a greater understanding of genetic relationships between groups of organisms. The evolution of proteins such as LDH provides an advantage over morphological characteristics in taxonomic studies in that these molecules may be less subject to gradual genetic change. However, morphology and behavior are linked to biochemistry. An incorporation of morphological, physiological, and biochemical methods gives the clearest and most accurate picture of taxonomy (Defalco, 1942).

The objective of this study was to compare the amount and pattern of LDH isozyme in eight species of fringillid birds. A disc electrophoresis unit was used for the separation of proteins due to their difference in charge. It has the advantage, as

opposed to starch block electrophoresis used by Fine and Costello (1962) and Bailey and Wilson (1968) of high resolution in brief runs. Only small quantities of protein are required, due to the fact that dilute protein samples are concentrated into discs of ten microns thickness at the beginning of the run before separation (Ornstein and Davis, 1961). If equal protein concentrations are subjected to electrophoresis under standard running conditions, a fair comparison of eight species of fringillid birds can be made.

MATERIALS AND METHODS

COLLECTING FRINGILLID BIRDS

All birds were caught with mist nets set on the East side of Rocky Butte, Multnomah County, Oregon, between 15 July 1970 and 15 August 1970. A 12 ft. x 6 ft. net was set in an open field surrounded by a Douglas fir and mixed deciduous forest. A second 8 ft. x 3 1/2 ft. net of smaller mesh was set as a backup net approximately 8 ft. behind the first. The second net could catch smaller birds, and if the birds avoided the first net they might become entangled in the second. The method was essentially the same as that of Johns (1963). As suggested by Martin (1969), care was taken not to trample feeding and netting areas in the field. The birds were captured when berries and other fruits were abundant. Traps utilizing food as a bait were useless during this time. Care was taken not to over-chloroform captured birds in order to avoid liver damage. The dead birds were weighed and frozen. Location of capture recorded. Capture data is summarized in Table I.

TABLE I. Capture data for eight species of fringillid birds.

All birds were collected on the East side of Rocky Butte, Portland Oregon.

Date of Capture:	Scientific names:	Common name and sex:
7/15/70	<u>Melospiza melodia</u>	male song sparrow
7/21/70	<u>Carpodacus mexicanus</u>	male house finch
8/1/70	<u>Passerella iliaca</u>	male fox sparrow
8/1/70	<u>Junco oreganus</u>	male Oregon junco
8/7/70	<u>Pipilo erythrophthalmus</u>	male rufous-sided towhee
8/8/70	<u>Pheucticus melanocephalus</u>	male black-headed grosbeak
8/11/70	<u>Hesperiphona vespertina</u>	male evening grosbeak
8/15/70	<u>Zonotrichia leucophrys</u>	male white-crown sparrow

METHODS FOR LACTIC DEHYDROGENASE STUDY

Chemicals purchased from Sigma Chemical are as follows; glycine, phenazene methosulfate, diphosphopyridine nucleotide, and potassium ferricyanide. Chemicals purchased from Mallinckrodt are as follows; potassium tartrate, copper sulfate, and sodium hydroxide. Chemicals purchased from Baker Chemical are as follows; glacial acetic acid and N, N' methylenebisacrylamide. Matheson Coleman and Bell chemicals were purchased as follows; 2-amino-2 (Hydroxymethyl)-1,3-propanediol (Tris), ammonium persulfate, ammonium sulfate, hydrochloric acid, acrylamide, bromphenol blue, trichloroacetic acid, riboflavin, N,N,N',N'tetramethylethylenediamine. Folin reagent was purchased from Uni-Teck. A Buchler electrophoresis polyanalyst unit was used with a current-regulated power supply.

Birds were thawed and liver, heart, and breast muscle were removed and minced separately. Equal volumes of each tissue were added to five volumes of 0.25 M sucrose and placed in a Sorvall Omnimixer at a setting of 7 for one minute. This mixture was then homogenized in a Potter-Elvehem glass homogenizer and centrifuged at 30,000 X g for 30 minutes at 0 C. The supernatant was poured off and frozen at -20 C.

Lactic dehydrogenase was partially purified as follows. Ammonium sulfate was added to 40% saturation (w/v). The solution was centrifuged at 30,000 X g for 10 minutes and the pellet discarded. Ammonium sulfate was added to the supernatant to give 60% saturation (w/v).

The solution was allowed to equilibrate for 15 minutes and centrifuged at 30,000 X g for 10 minutes. The 60% ammonium sulfate pellet was suspended in two pellet-volumes of the 60% ammonium sulfate supernatant. This solution was dialyzed against 500 volumes of 5 mM Tris-HCL buffer (pH 8.5) for 24 hours at 6.6 C.

Protein concentration was determined by the method given by Lowry et al. (1951). Using bovine serum albumin as the standard, unknown protein concentrations could be calculated (Figure 1). Each sample was adjusted to 250 ug/ml of protein prior to electrophoresis. Each electrophoresis run lasted 45 minutes after bromophenol blue tracer reached the end of the gel.

The method of disc electrophoresis was essentially the same as that done by Ornstein and Davis (1961) with the following changes. Protein samples were added to a 40% sucrose solution. Each sample was added with a long pipet directly to the top of the stacking gel. The whole unit was run at 6.6 C. The upper buffer contained 5.16 g Tris, 3.48 g glycine and water to 1 liter. The pH at 22 C was 8.9 and at 0 C, 9.64. The lower buffer contained 14 g Tris, 60 ml 1 N HCL and 940 ml of water. The pH of the lower buffer at 22 C was 8.07 and at 0 C, 8.84. Duplicate gels 3 cm in length were prepared from each protein sample. Protein bands were stained with Coomassie blue according to the procedure of Chrambach et al. (1967). This procedure permitted comparison of amounts of isozyme in different samples. Protein bands of LDH were detected by the nitro-blue tetrazolium (NBT) staining method of Fine and

Costello (1962). This method is extremely sensitive, but the amount of LDH activity cannot be quantified using this procedure. Gel tracings were recorded on a Densicord recording electrophoresis densitometer using filter no. 610 and no. 5 response. Comparisons between the tracings of the protein and NBT stains were made by aligning the stacking gels. Lactic dehydrogenase isozymes were labeled and a comparison of sample was made from sets of tracings.

Individual variation was found by McCabre (1952) to prevent distinction of species by egg white protein samples. McCabre also found individual variation in serum protein patterns if the birds tested were not healthy, or differed in age and sex. The birds used in this study were males, had undergone their post-juvenal molt, and appeared to be healthy. Under the conditions of this experiment, protein samples of two song sparrows had identical LDH patterns.

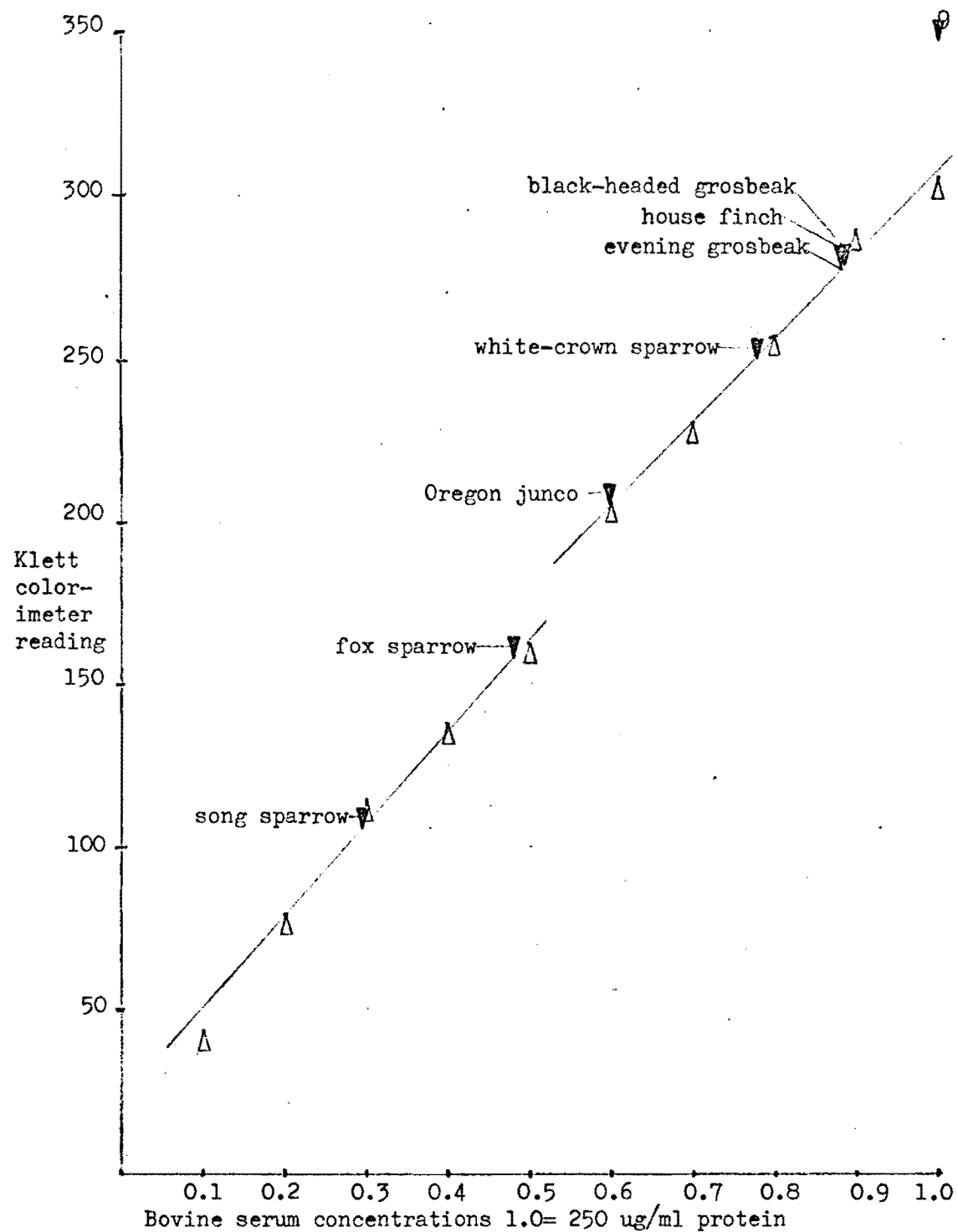


Figure 1. Standard curve for determining protein concentration of samples for electrophoresis; Δ = bovine serum plots; ▽ = bird protein sample.

RESULTS

Qualitative data are often more useful than quantitative data in biochemical taxonomic studies (Sibley and Johnsgard, 1959).

A female house finch was used in the study of lactic dehydrogenase isozyme migration. Gels were removed from the electrophoresis unit at intervals of 30, 75, and 120 minutes after the tracer reached the bottom of the gel. At 120 minutes splitting occurred in the isozyme patterns. The homogenate had been frozen a number of times to -20°C . The same results were found by Vesell and Brody (1968). Results are shown on Figures 2, 3, and 4.

The H_4 isozyme appeared on the NBT stain as a massive broad peak with very little protein detected on the Coomassie blue tetrazolium stain.

The results of the taxonomic experiments on eight species of fringillid birds are shown in Figures 5 through 12. The song sparrow and the fox sparrow are grouped independently from the rest of the birds studied due to the following characteristics: the five isozymes did not migrate as far; there is higher amounts of H_4 isozyme; H_2M_2 isozyme was very small or missing; this group lacks the sharp partition between H_2M_2 and H_3M_1 isozymes.

The black-headed grosbeak and evening grosbeak are loosely grouped together due to the large H_3M_1 isozyme activity on the NBT stain with very little protein existing. The rufous-sided towhee, white-crown sparrow, and the Oregon junco are linked with the black-headed grosbeak and the evening grosbeak by having very little H_1M_3 isozyme

with a large amount of NBT activity. Unlike the latter group of birds, the house finch has a moderate amount of the H_1M_3 isozyme.

Representative gels of several species are shown in Figure 13. Figure 14 summarizes the data from Figures 5 through 12.

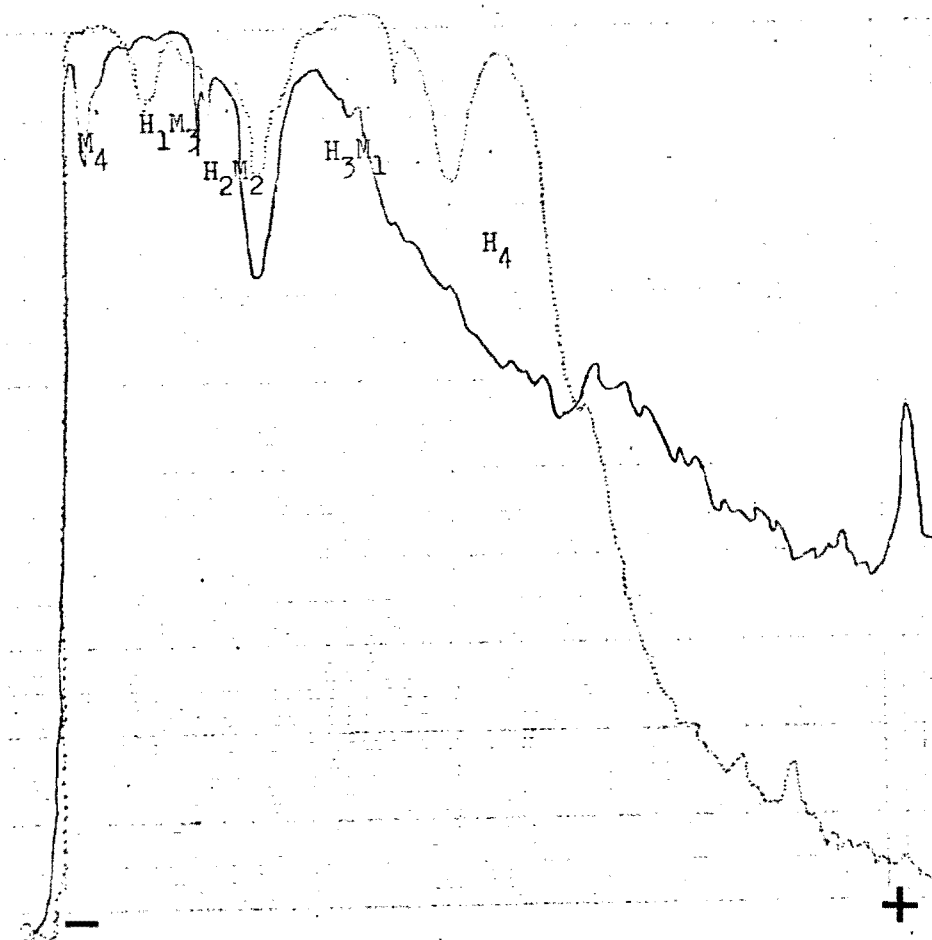


Figure 2. Pattern of LDH isozyme migration after 30 minutes past the tracer. Solid line represents protein stain (Coomassie blue); dotted line represents activity stain (NBT). A female house finch was used as a sample. Migration of the proteins is from left to right.

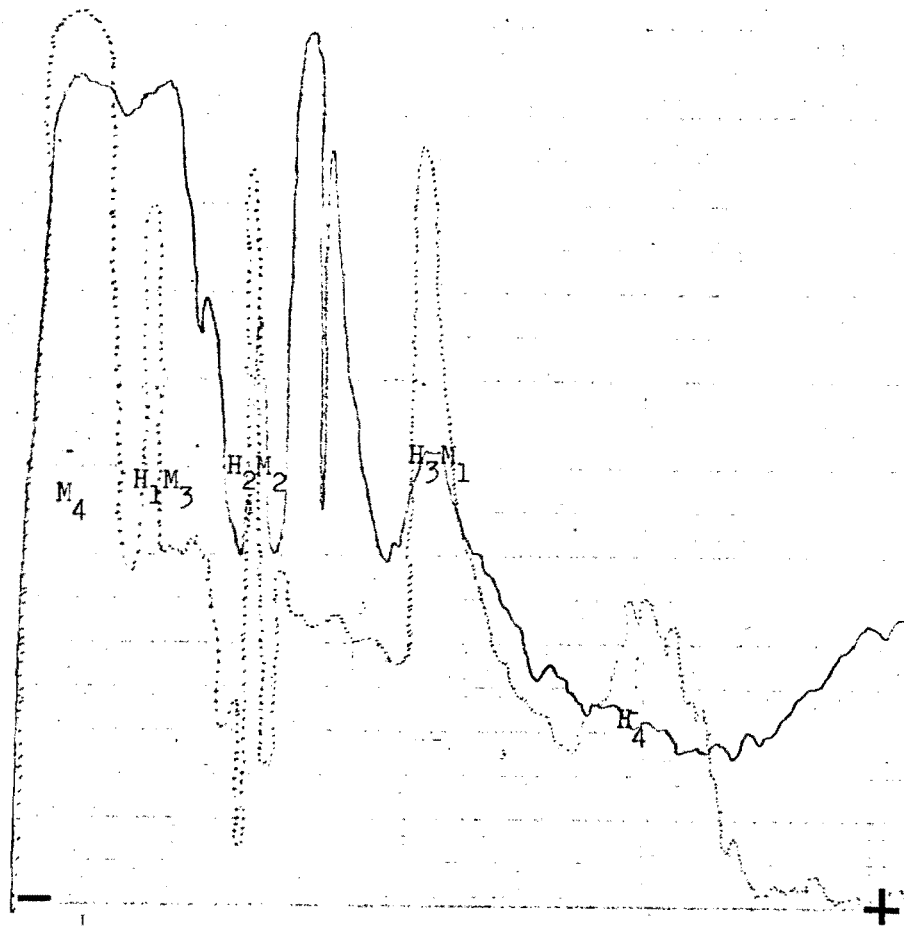


Figure 3. Pattern of LDH isozyme migration after 75 minutes past the tracer. Solid line represents protein stain (Coomassie blue); dotted line represents activity stain (NBT). A female house finch was used as a sample. Migration of the proteins is from left to right.

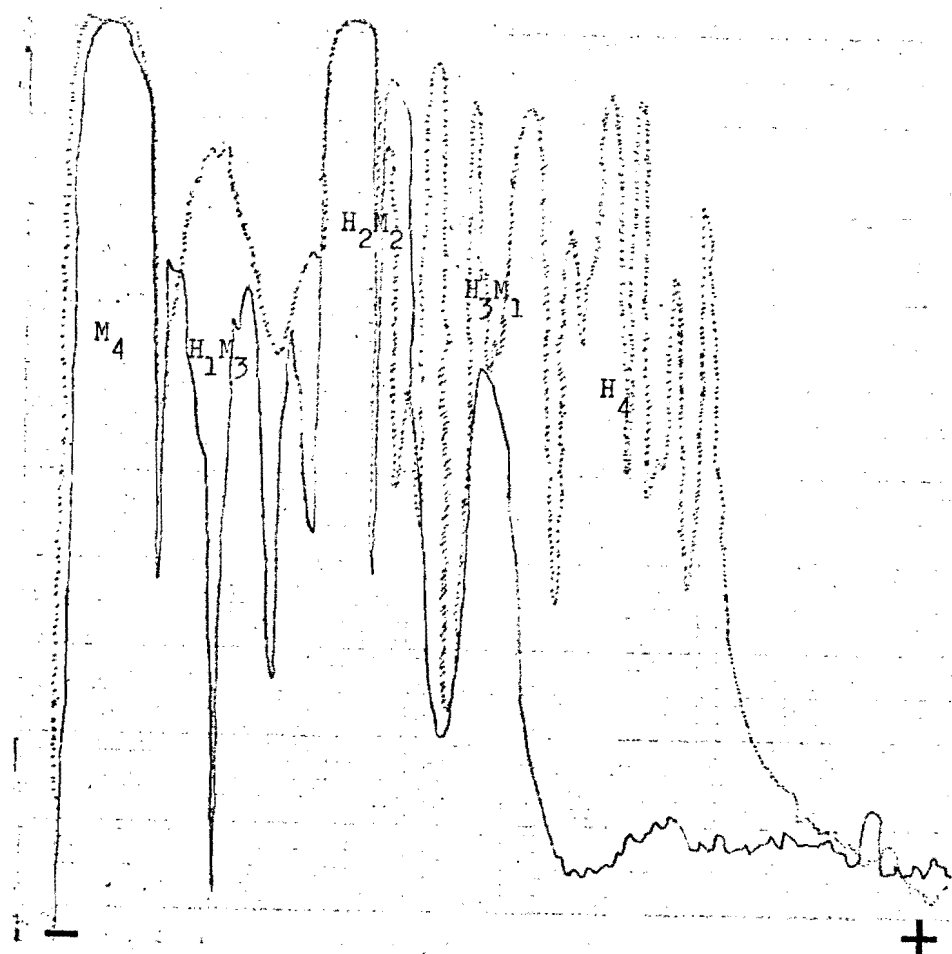


Figure 4. Pattern of LDH isozyme migration after 120 minutes past the tracer. Solid line represents protein stain (Coomassie blue); dotted line represents activity stain (NBT). A female house finch was used as a sample. Migration of the proteins is from left to right. Note the splitting of H_3M_1 and H_4 isozymes due to the freezing of the homogenate.

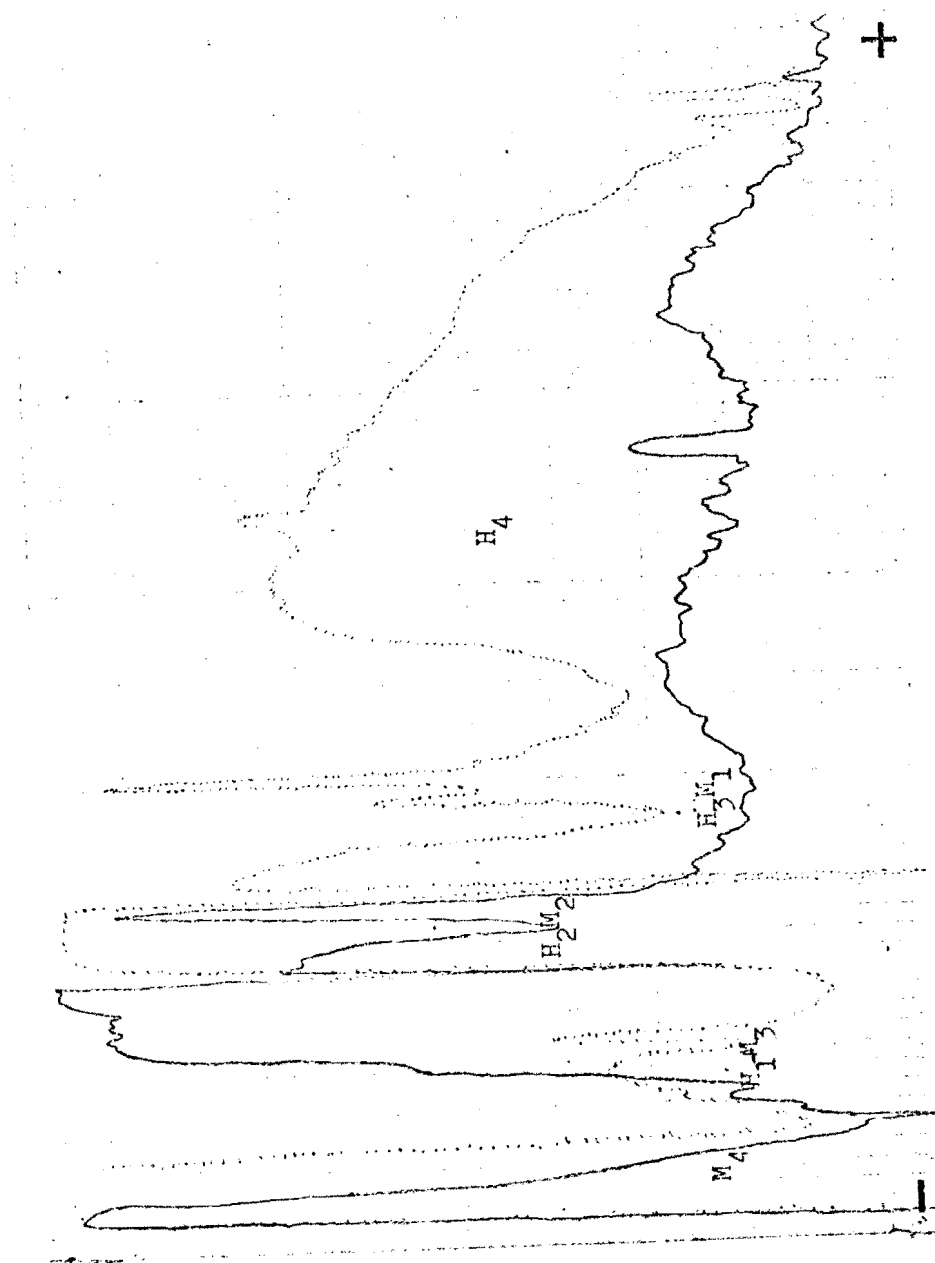


Figure 5. Pattern of LDH isozyme densitometer tracings of a male black-headed grosbeak. Solid line represents protein stain (Coomassie blue); dotted line represents activity stain (NBT).

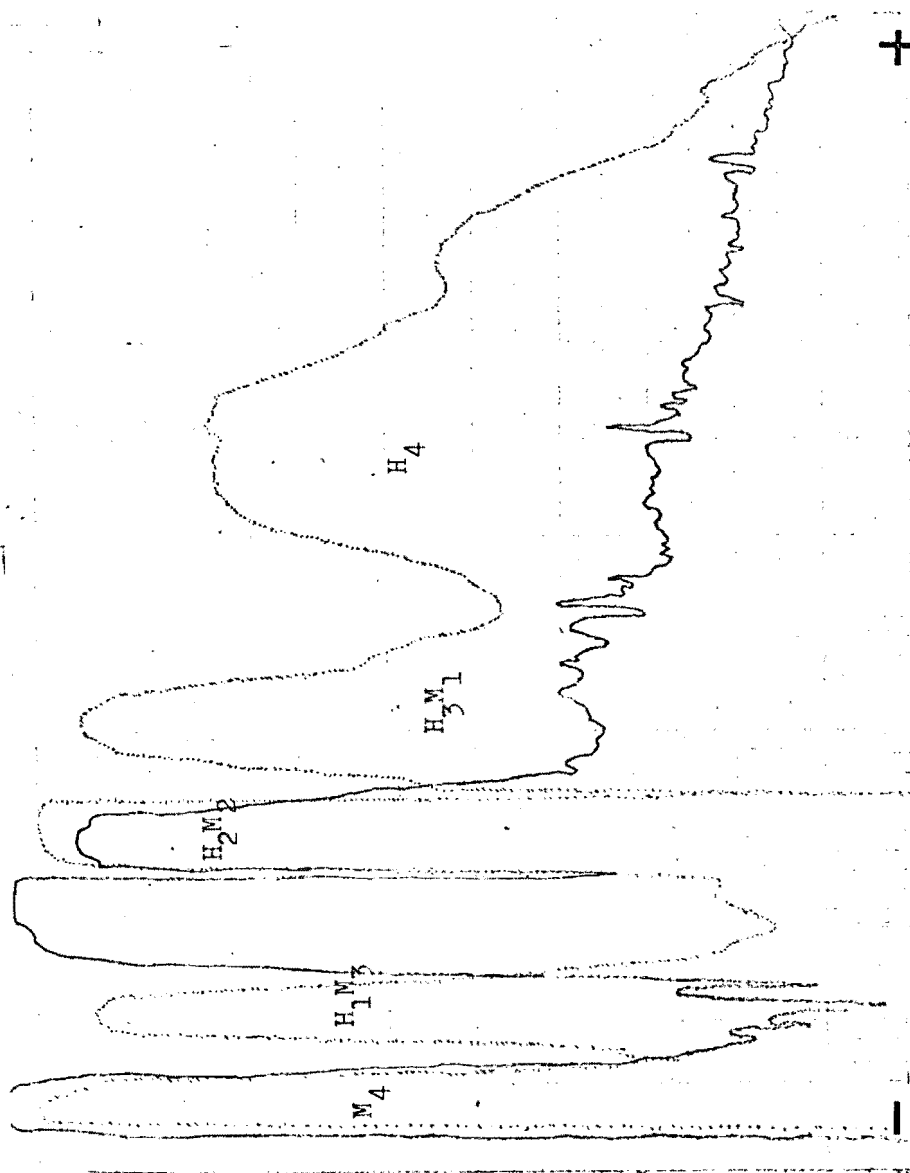


Figure 6. Pattern of LDH isozyme densitometer tracings of a male evening grosbeak. Solid line represents protein stain (Coomassie blue); dotted line represents activity stain (NBT).

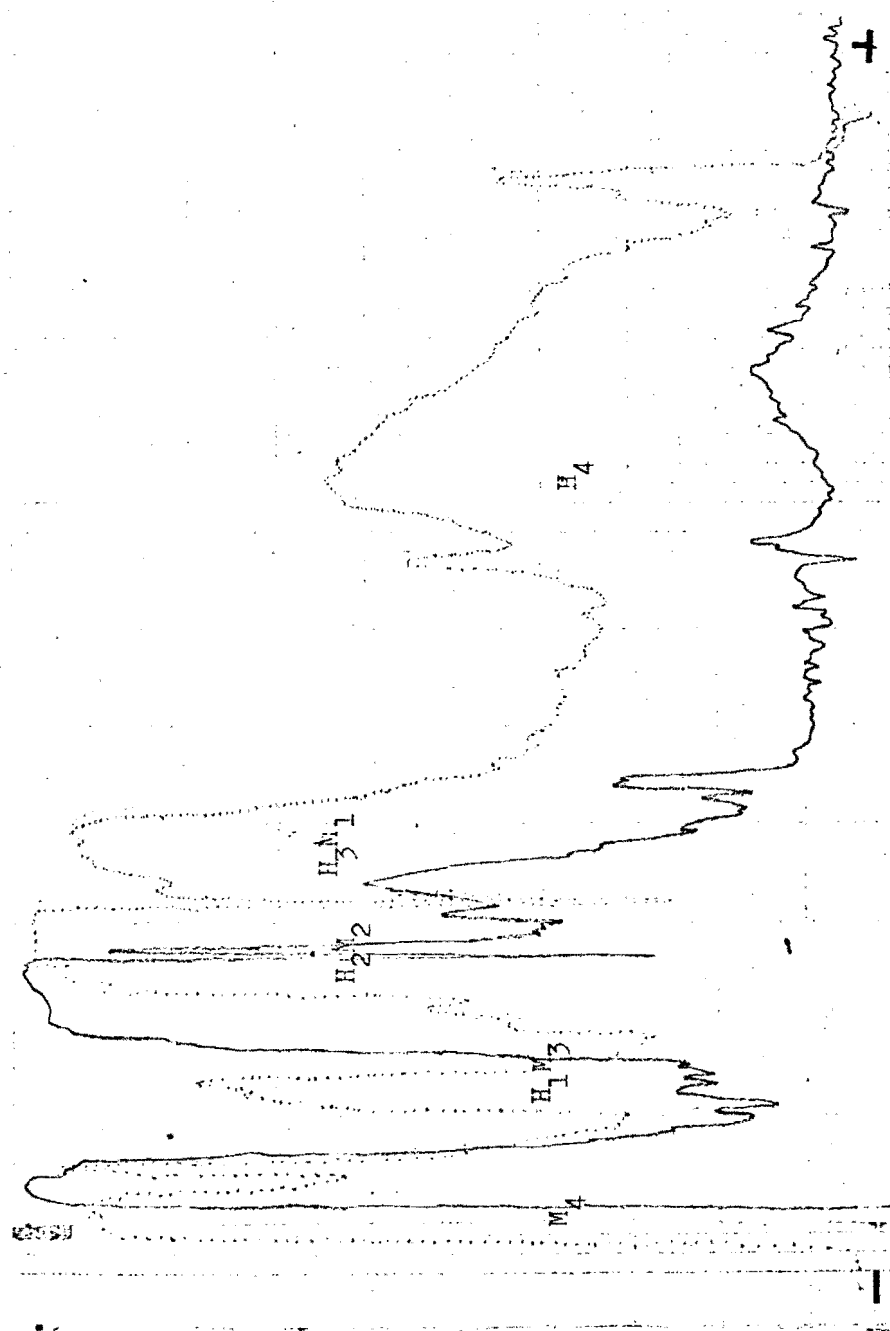


Figure 7. Pattern of LDH isozyme densitometer tracings of a male Oregon junco. Solid line represents protein stain (Coomassie blue); dotted line represents activity stain (NBT).

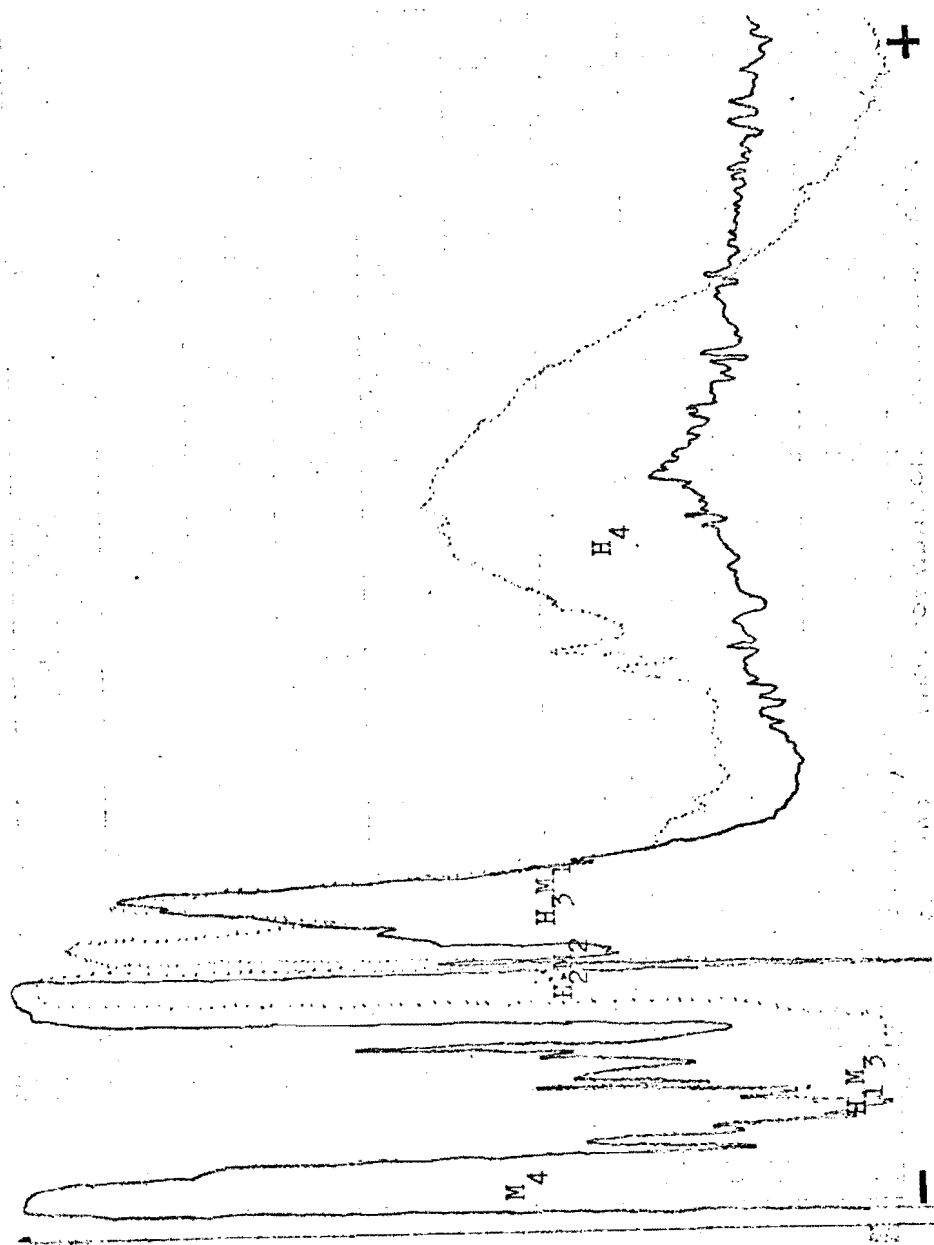


Figure 8. Pattern of LDH isozyme densitometer tracings of a male rufous-sided towhee. Solid line represents protein stain (Coomassie blue); dotted line represents activity stain (NBT).

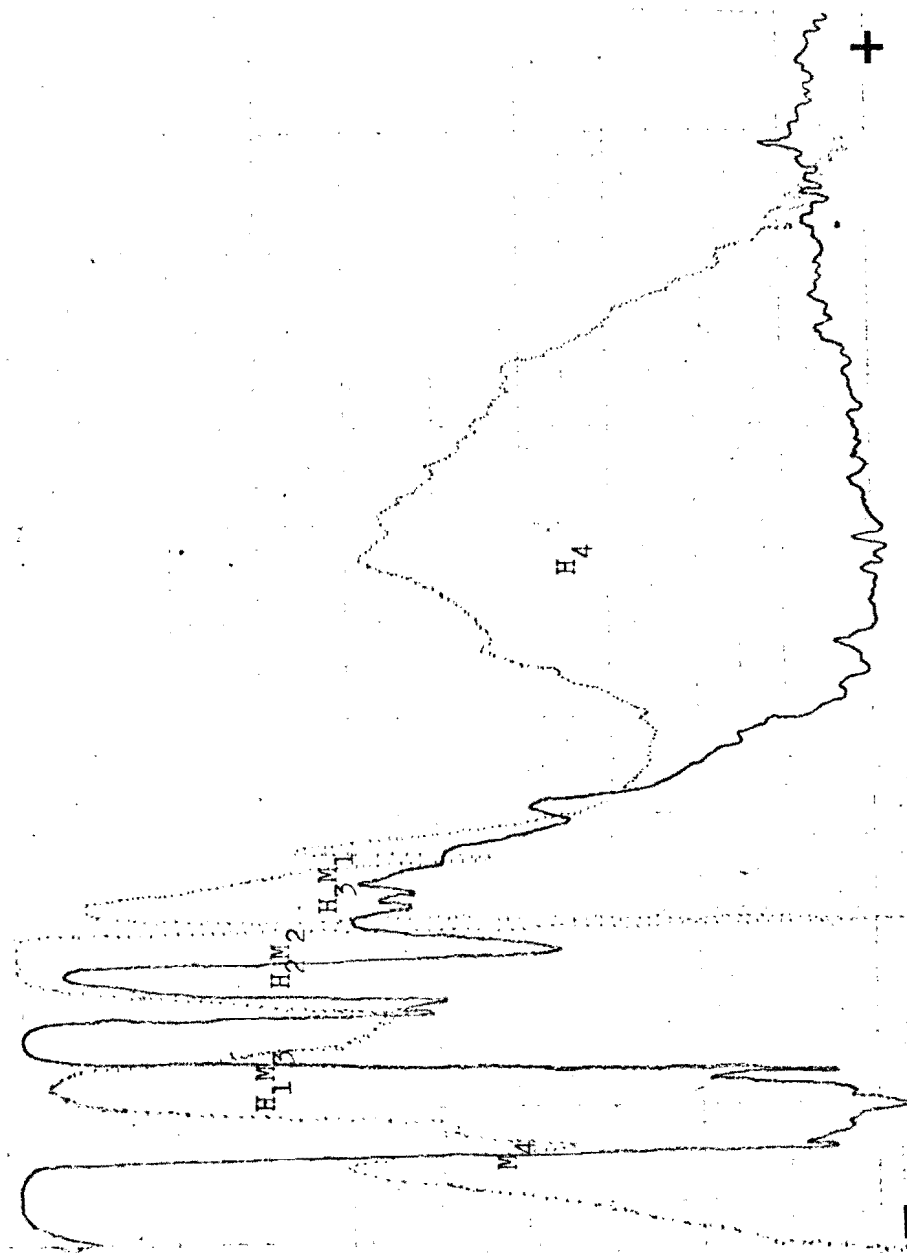


Figure 9. Pattern of LDH isozyme densitometer tracings of a male white-crown sparrow. Solid line represents protein stain (Coomassie blue); dotted line represents activity stain (NBT).

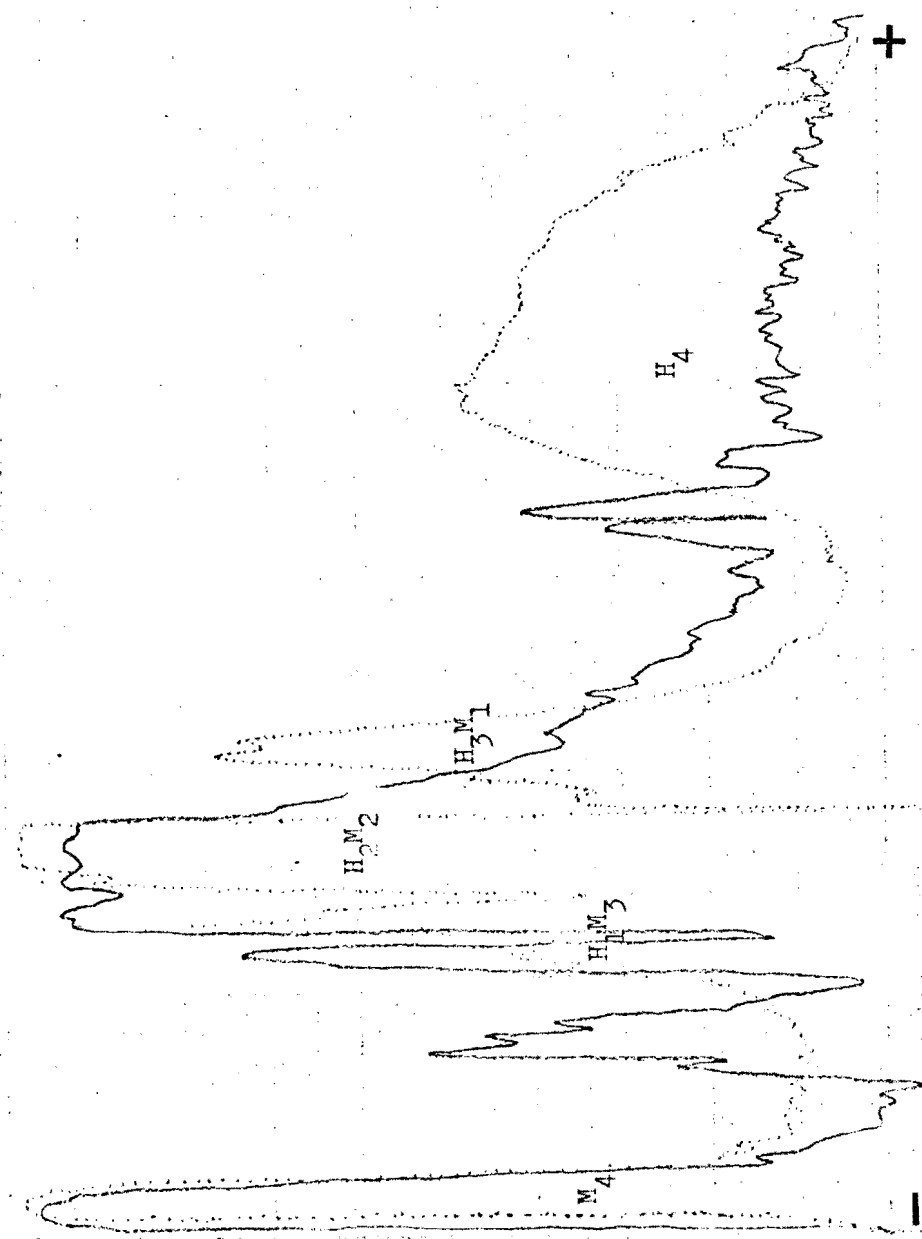


Figure 10. Pattern of LDH isozyme densitometer tracings of a male house finch. Solid line represents protein stain (Coomassie blue); dotted line represents activity stain (NBT).

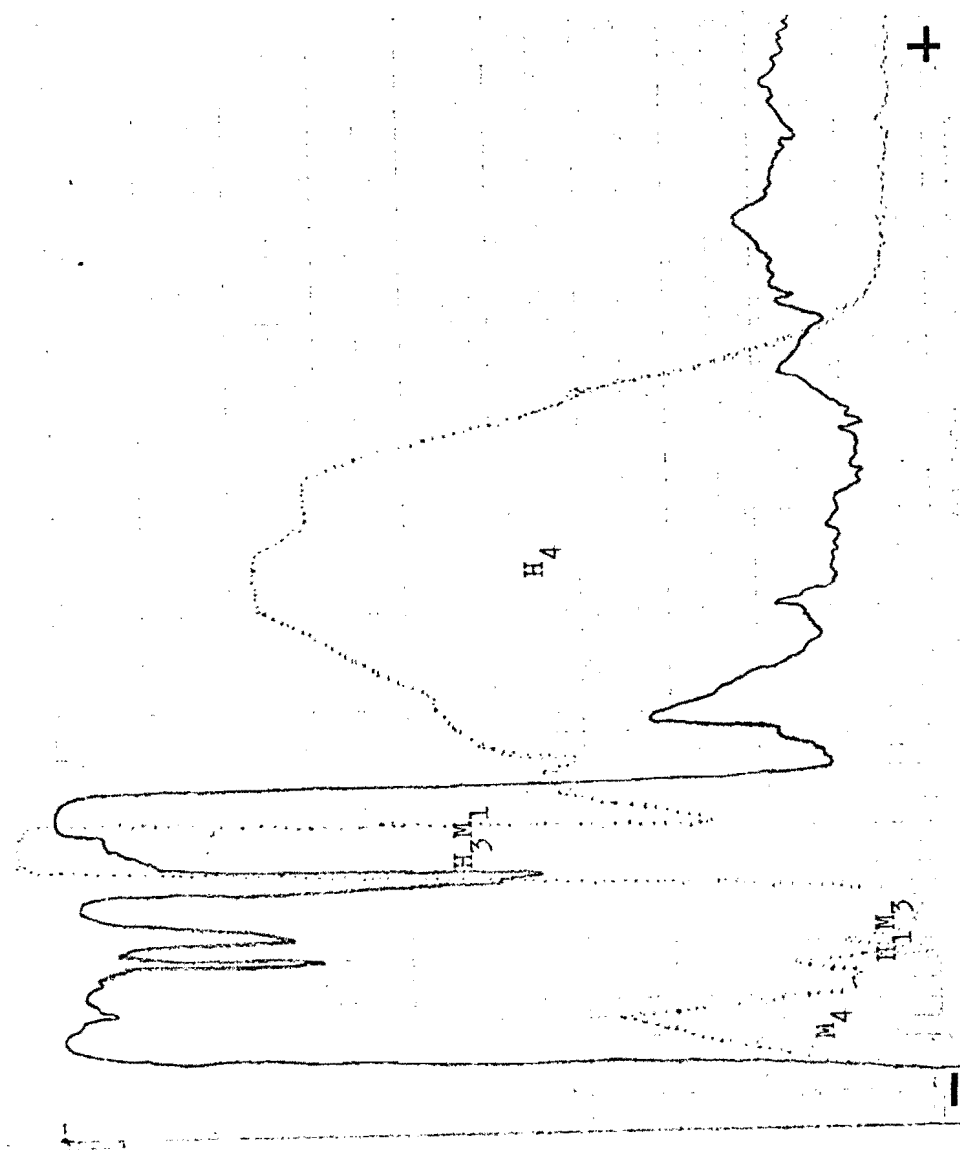


Figure 11. Pattern of LDH isozyme densitometer tracings of a male song sparrow. Solid line represents protein stain (Coomassie blue); dotted line represents activity stain (NBT).

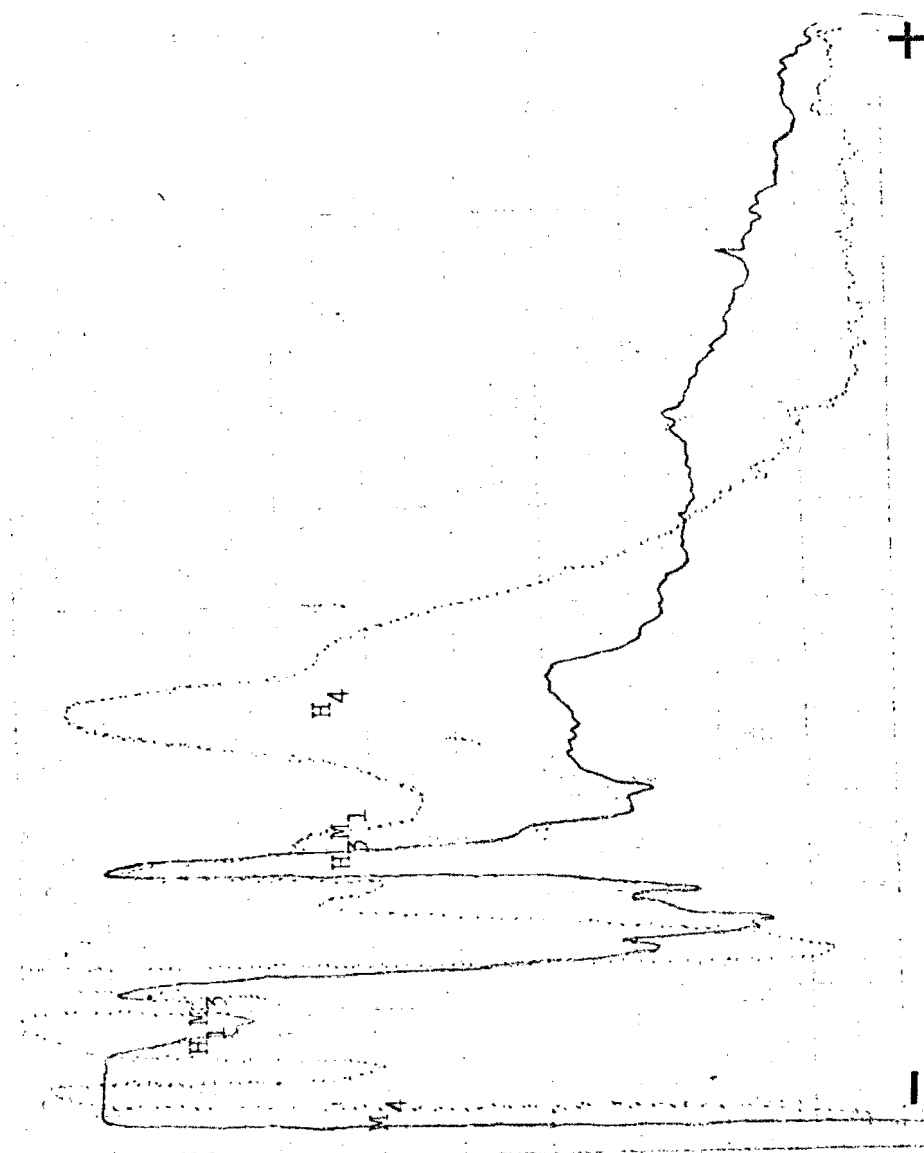


Figure 12. Pattern of LDH isozyme densitometer tracings of a male fox sparrow. Solid line represents protein stain (Coomassie blue); dotted line represents activity stain (NBT).

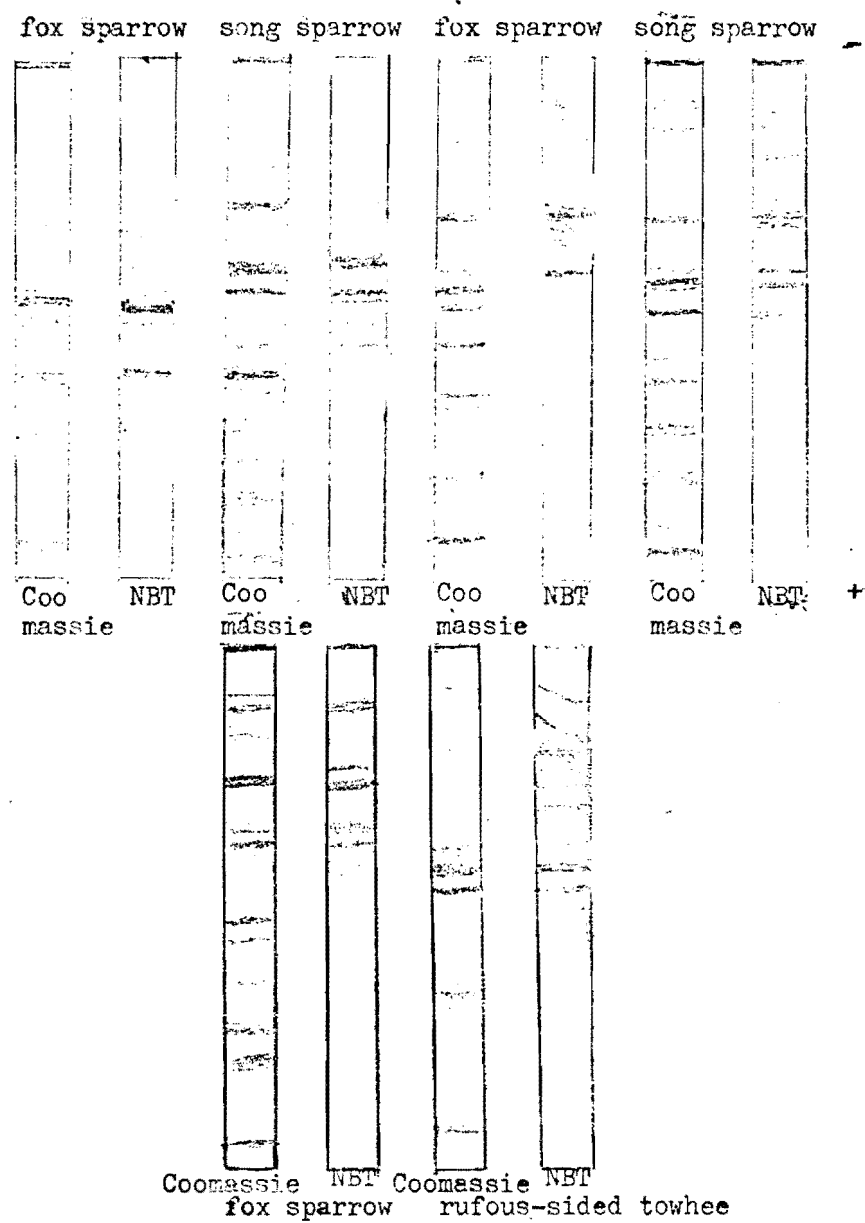


Figure 13: LDH isozyme gel tracings.

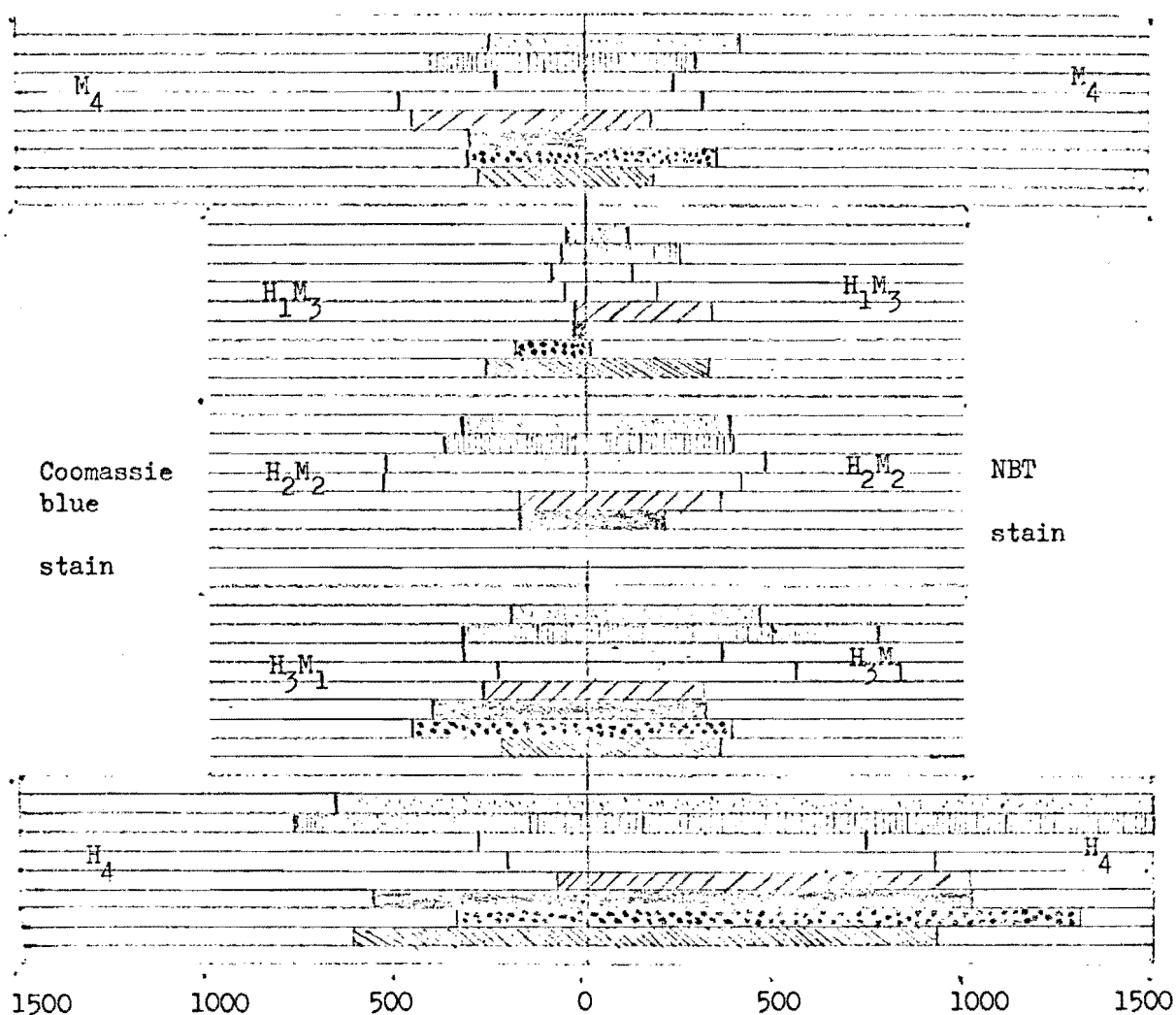










Figure 14. Summary of data of eight species of fringillid birds is

the area under the curve of LDH isozyme densitometer tracings.

Units used are proportional;  = black-headed grosbeak,  = evening grosbeak,  = house finch,  = Oregon junco,  = white-crown sparrow,  = rufous-sided towhee,  = song sparrow,  = fox sparrow.

DISCUSSION

Four methods of classification for fringillid birds have been suggested. Beecher (1953) utilized homologies of jaw-structure. Tordoff (1954) employed the structure of horny and bony palates. Nelson (1954) used comparative osteology. Stallcup (1955) relied on leg musculature and immunological serum analysis. Table II summarizes the classification resulting from these findings. Tordoff, Nelson, and Stallcup's methods are discussed at length by Stallcup (1955). Boyden (1942) has suggested that morphological features, as opposed to biochemical ones, are more variable and change faster due to natural selection pressures. Morphological features relate directly to behavior such as feeding habits, as seen in Darwin's study of finches (Sibley, 1957). These finches underwent considerable adaptive radiation from one ancestral stock in a relatively short period of time (Lack, 1939). Newly emerging species can be a problem to the morphological taxonomist due to convergence and parallelism. It was not possible for Stallcup (1955) to do every immunological experiment needed to show genetic relationships. If eight species of fringillid birds were studied, there would be 64 different experiment needed to show all possible relationships.

The evidence from my studies suggest a classification most like that of Stallcup (1955). He places the fox and song sparrows in the family Carduelidae, subfamily Estrildinae. The evening grosbeak and the black-headed grosbeak were placed in the family Carduelidae,

subfamily Carduelinae. However, Stallcup (1955) places the juncos and white-crown sparrow in the family Fringillidae, subfamily Richmondinae and the towhees in the family Fringillidae, subfamily Emberizinae. Lactic dehydrogenase isozyme patterns suggest that the juncos, white-crown sparrow, and towhees are best assigned to the family Carduelidae, subfamily Richmondinae.

It has been found that enzymes of equal molecular weight can take up different amounts of dye (Hansl, 1964). This might account for the tremendous binding of NBT stain with very little amounts of H_4 isozyme. Since H_4 isozyme is known to inhibit pyruvate to lactate, any large amount of the enzyme would severely block the metabolic pathway.

The splitting of the five isozymes under conditions given in the results are probably due to partial breakage of the polypeptide chains caused by repeated freezing and thawing. Under natural conditions, in which no splitting occurs, the four polypeptide subunits that make up each isozyme will remain a unit.

TABLE II. Comparison of scheme of classification of eight species
of fringillid birds.

Here Proposed:	Stallcup:	Tordoff:	Beecher:	Nelson:
Family Card- elidae	Family Cardu- elidae	Family Plo- ceidae	Family Estr- ildidae	Family Thr- aupidae
Subf. Est- rildinae	Subf. Est- rildinae	Subf. Estril- dinae	<u>Carpodacus</u> <u>mexicanus</u>	Subf. Rich- mondeninae
<u>Carpodacus</u> <u>mexicanus</u>	<u>Carpodacus</u> <u>mexicanus</u>	<u>Carpodacus</u> <u>mexicanus</u>	Family Thr- aupidae	<u>Zonotrichia</u> <u>leucophrys</u>
Subf. Card- uelinae	Subf. Cardu- uelinae	Subf. Cardu- uelinae	Subf. Pyr- rhuloxiinae	<u>Junco</u> <u>oreganus</u>
<u>Pheucticus</u> <u>melanocephalus</u>	<u>Pheucticus</u> <u>melanocephalus</u>	<u>Pheucticus</u> <u>melanocephalus</u>	Subf. Car- duelinae	Family Frin- gillidae
<u>Hesperiphorna</u> <u>vespertina</u>	<u>Hesperiphorna</u> <u>vespertina</u>	<u>Hesperiphorna</u> <u>vespertina</u>	<u>Pheucticus</u> <u>melanocephalus</u>	Subf. Ember- izinae
Subf. Rich- mondeninae	Family Frin- gillidae	Family Frin- gillidae	<u>Hesperiphorna</u> <u>vespertina</u>	<u>Pheucticus</u> <u>melanocephalus</u>
<u>Junco</u> <u>oreganus</u>	Subf. Rich- mondeninae	Subf. Rich- mondeninae	Subf. Thraup- inae	<u>Hesperiphorna</u> <u>vespertina</u>
<u>Pipilo</u> <u>erythrophthalmus</u>	<u>Zonotrichia</u> <u>leucophrys</u>	<u>Zonotrichia</u> <u>leucophrys</u>	Family Par- ulidae	
<u>Zonotrichia</u> <u>leucophrys</u>	<u>Junco</u> <u>oreganus</u>	<u>Junco</u> <u>oreganus</u>	Subf. Paru- linae	
Family Fring- illidae	Subf. Thrau- pinae	Subf. Thrau- pinae	<u>Zonotrichia</u> <u>leucophrys</u>	
Subf. Ember- izinae	Subf. Ember- izinae	Subf. Frin- gillidae	<u>Junco</u> <u>oreganus</u>	

TABLE II. Cont.

Here	Stallcup:	Tordoff:	Beecher:	Nelson:
Proposed:				
(Subf. Frin- gillinae)	(Subf. Frin- gillinae)	(including Emberizinae and Geospiz- inae)	Subf. Emb- erizinae	(including Geospizinae)
(Subf. Geo- spizinae)	(Subf. Geo- spizinae)			
<u>Passerella</u> <u>iliaca</u>	<u>Passerella</u> <u>iliaca</u>	<u>Passerella</u> <u>iliaca</u>	<u>Passerella</u> <u>iliaca</u>	<u>Passerella</u> <u>iliaca</u>
<u>Melospiza</u> <u>melodia</u>	<u>Melospiza</u> <u>melodia</u>	<u>Melospiza</u> <u>melodia</u>	<u>Melospiza</u> <u>melodia</u>	<u>Melospiza</u> <u>melodia</u>
	<u>Pipilo</u> <u>erythroph-</u> <u>thalmus</u>	<u>Pipilo</u> <u>erythroph-</u> <u>thalmus</u>	<u>Pipilo</u> <u>erythroph-</u> <u>thalmus</u>	<u>Pipilo</u> <u>erythroph-</u> <u>thalmus</u>

CONCLUSIONS

If the birds sampled are of the same sex, age, and appear to be healthy, variability within species or subspecies of LDH isozyme patterns is substantially reduced. A fair taxonomic comparison can be made if the disc electrophoresis running time, temperature, power supply, concentration of protein and proportion of tissue components are held constant.

My evidence suggest the following changes in classification from those employed by Stallcup (1955). The rufous-sided towhee should be removed from the family Fringillidae, subfamily Emberizinae, and placed in the family Carduelidae, subfamily Richmondininae. The Oregon junco and the white-crown sparrow should be removed from the family Fringillidae, subfamily Richmondininae, and placed in the family Carduelidae, subfamily Richmondininae.

If tissue homogenate has been frozen a number of times before electrophoresis and allowed to proceed 120 minutes past the bromphenol tracer, then splitting of H_4 and H_3M_1 isozymes occurs.

A small amount of H_4 isozyme produces a large amount of enzymatic activity.

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